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Antioxidant action of estrogens in rat hepatocytes

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The *in vitro* addition of 17ß-estradiol (0-100 µM) to isolated rat hepatocytes efficiently prevented cellular lipid oxidation induced by the Fe(III)/ADP complex. 17ß-estradiol was found to be less effective than its metabolic derivative 2-hydroxyestradiol. The presence of specific inhibitors of cytochrome P450 activity significantly diminished the antioxidant capacity of estradiol. These observations support the hypothesis that estradiol, in the micromolar range, inhibits iron-induced lipid peroxidation in liver cells by diverting reducing equivalents from the peroxidative process to its own metabolism.

Key words: 17β-Estradiol, Antioxidant, Lipid peroxidation, Rat hepatocyte

Natural and synthetic estrogens have proved to be effective antioxidant agents in different *in vitro* systems (1, 5, 8, 15). However, the mechanism of the estrogen protective actions is not yet fully understood. Several proposals have been made to explain these effects. These include stabilization of membrane fluidity (16) and both radical-trapping and metal-chelating properties ascribed to their phenolic structures (1, 5, 8, 15).

17β-estradiol (E_2) was previously shown to be a good protector of microsomal lipid peroxidation initiated by NADPH and Fe(III)/ADP (11). To a lesser extent E_2 also prevented Fe(II)/ascorbate-induced lipid peroxidation. Interestingly, the protective effect of E_2 in the Fe(II)/ascorbate model was considerably increased under conditions where microsomal metabolism was favored (6).

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Abbreviations: DMSO, dimethylsulfoxide; E_2, 17β-estradiol; 2-OHE_2, 2-hydroxyestradiol; LDH, lactate dehydrogenase; MDA, malondialdehyde; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances.

In the endoplasmic reticulum of the liver, E_2 is mainly converted into 2- and 4-hydroxyestradiol by the action of a NADPH cytochrome P450-dependent monooxygenase system, which also mediates the initiation of NADPH-driven lipid peroxidation. The hydroxylative transformation of E_2 may thus interfere by competition with the peroxidation process. The present work was carried out to examine this possibility, using freshly isolated rat hepatocytes as the experimental model. Since the ability of estrogens to inhibit peroxidation has not yet been established in this *in vitro* system, we first examined the effects of E_2 and 2-OHE_2 on iron-induced lipid peroxidation in liver cells. The effects of E_2 on Fe(III)/ADP-induced lipid peroxidation were then studied in cells treated with specific inhibitors of cytochrome P450 activity.

Materials and Methods

Preparation and incubation of hepatocytes.– Liver cells were isolated from male Sprague-Dawley rats (180 g) as previously described (9). Hepatocytes (2 x 10^6 cells/mL) were incubated in Dulbecco's Minimum Essential Medium, pH 7.4, plus 10 mM Hepes and 2 % BSA at 37 °C under an atmosphere of 95 % O_2/5 % CO_2. After 20 min preincubation, peroxidation was initiated by the addition of 50 µM $FeCl_3$ and 1 mM ADP (previously complexed for at least 30 min), and the incubation continued for up to 4 h. E_2 and 2-OHE_2, dissolved in DMSO (at the final concentration of 0.1 %) were added during preincubation (10 min before the start of reactions). When inhibitors of cytochrome P450 (metyrapone and proadiphen) were used, they were added to cell suspensions at the start of the preincubation time. Other experimental details are described in the legend to the figures. At timed intervals, aliquots were removed for determination of both cell viability (LDH test) and lipid peroxidation (TBA test). In this case, trichloroacetic acid (10 % final concentration) was added to the samples to stop the reactions.

TBA assay.– Lipid peroxidation was measured in the supernatants of deproteinized cells by the TBA method (2). Lipid peroxidation was expressed as nanomoles of malondialdehyde (MDA) per million of cells, using the MDA extinction coefficient of 156 $mM^{-1}cm^{-1}$ at 532 nm. Neither the tested estrogens nor iron interfered with the TBA assay.

Statistical significance was determined by Student's *t* test for non-paired data.

Results and Discussion

The first aim of the present study was to evaluate the antioxidant activities of E_2 and 2-OHE_2 in hepatocytes stimulated with Fe(III)/ADP. Isolated cells represent an excellent biological model of *in vitro* study of lipid peroxidation, since the cells maintain their functional integrity and their defence systems remain active against oxidative challenge. TBARS production was measured as indicator of lipid peroxidation and the intracellular LDH release into the medium as a marker of cellular injury.

Figures 1 and 2 show the time course of cell peroxidation and the effects of E_2 and 2-OHE_2, respectively, on this process. The addition of Fe(III)/ADP to hepatocyte incubates increased the formation of TBARS with time. Cell viability, which was 95.5 ± 1.3 (N = 7) at zero time, decreased less than 10% over the 4 h incubation, and was not affected by the presence of iron. E_2 (0-100 µM) significantly inhibited peroxidation in a dose-depen-

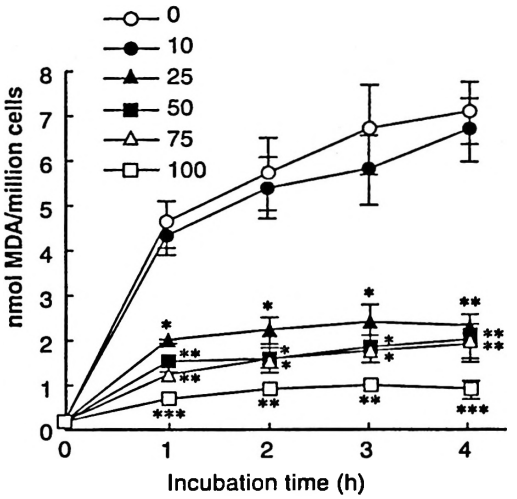

Fig. 1. *Effect of E2 on lipid peroxidation induced by Fe(III)/ADP.*
Hepatocytes were incubated with Fe(III)/ADP (50 µM/1 mM) and E2 at the indicated µM concentrations as described in the text. Values are the means ± S.E. of 3 independent experiments with different hepatocyte preparations. *p<0.05; **p<0.01; ***p<0.005

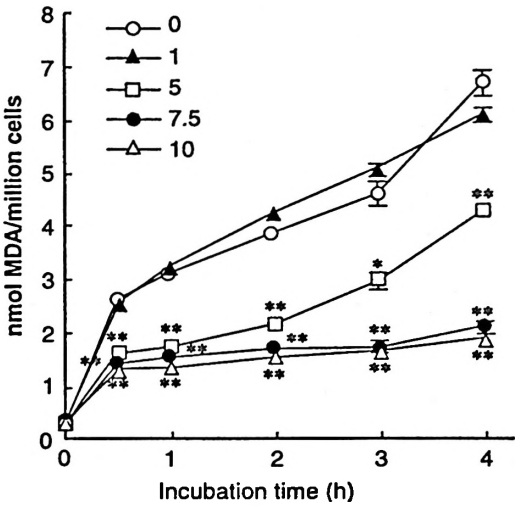

Fig. 2. *Effect of 2-OHE2 on lipid peroxidation induced by Fe(III)/ADP.*
2-OHE2 was assayed at the indicated µM concentrations. Incubations were performed as described in the legend to figure 1. Values are the means ± S.E. of N = 3. *p<0.01; **p<0.005.

dent manner (fig. 1). 2-OHE$_2$ (0-10 µM) had a potent inhibitory effect. Compared to E$_2$, the catecholestrogen was about 3-fold more efficient (fig. 2).

Whether E$_2$ needed to be present during the inductive stage of peroxidation to exert its effects was also investigated. In these experiments cells were pretreated with 100 µM E$_2$ for different times (5-30 min), they were washed twice and resuspended in fresh medium, followed by peroxidation initiated by the addition of Fe(III)/ADP (80 µM/1.6 mM). A 5 min E$_2$ pretreatment was found to be enough to produce a substantial decrease in TBARS production (fig. 3). Longer preincubation times with the hormone (15 and 30 min) resulted in greater inhibitions (fig. 3). Control values (DMSO pretreated hepatocytes) at each time were independent of the preincubation periods with the solvent.

The possible relationships between the E$_2$ antioxidant activity and the microsomal metabolism of the hormone were investigated, through studies with specific inhibitors (proadiphen, metyrapone) of the P450 cytochromes which participate in the E$_2$ hydroxylation (4, 13, 14). Effectors were added to hepatocyte suspensions and incubated with E$_2$ (0-100 µM) before the addition of the pro-oxidant. As fig. 4 shows, cell treatments with P450 cytochrome inhibitors diminished the preventive effects of E$_2$ on lipid peroxidation. In the case of proadiphen (100 µM) decreases were significant for most E$_2$ concentrations. However, in the case of metyrapone (500 µM), only at the highest E$_2$ concentration were the decreases significant.

Oxidative metabolism may play a role in the protective action of E$_2$. In the above experiments, an Fe(III)/ADP complex was used to initiate peroxidation with the consequent entrance of iron into the cell

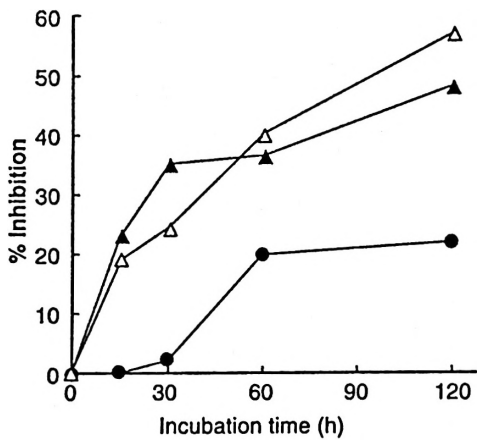

Fig. 3. *Effect of several E2 pretreatments on lipid peroxidation induced by Fe(III)/ADP.*
Cells were pretreated with 100 μM E2 for 5 (●), 15 (Δ), and 30 (▲) min. After preincubation, cells were washed and resuspended in fresh medium. Peroxidation was initiated by the addition of Fe(III)/ADP (80 μM/1.6 mM). Results are expressed as percentages of inhibition of peroxidation in controls (cells pretreated with the vehicle) at the same incubation times. Values are the means of triplicates from a type experiment.

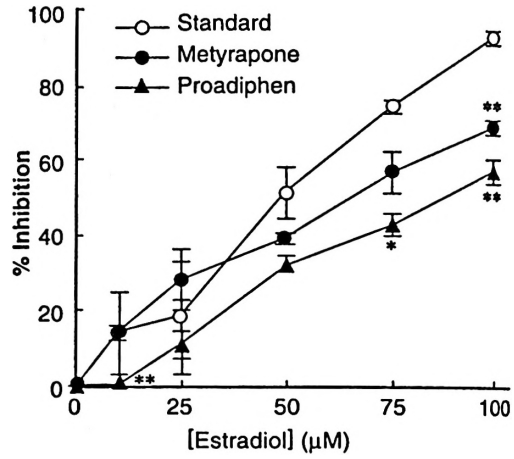

Fig. 4. *Effects of metyrapone (500 μM) and proadiphen (100 μM) on the antioxidant action of E2 against lipid peroxidation induced by Fe(III)/ADP.*
Cells were incubated with the inhibitors of cytochrome P450 and the indicated concentration of E2 under the conditions described. Results are expressed as percentages of inhibition of peroxidation in incubates without E2. Values are the means ± S.E. of 3 independent experiments with different hepatocyte preparations. *p<0.01; **p<0.005.

and its further reduction accomplished by NADPH:cytochrome P450 reductase (3). Ferrous ion promotes the formation of both reactive oxygen species, *via* the Fenton reaction, and Fe(II)/Fe(III) complexes that stimulate peroxidation. The interaction of the enzymatic iron reduction with E_2 hydroxylation may arise from electron transference from NADPH to specific isoformes of cytochrome P450, this transference being catalyzed by the reductase system. Accordingly, E_2 and other estrogens have been recently shown to reduce the rate of NADPH-driven reduction of Fe(III) in rat liver microsomes (12).

The mechanism by which estrogens inhibit lipid peroxidation seems to be rather complex, although it has not yet been clarified. Estrogens interact with propagation reactions of the process and with reactive species capable of initiating lipid peroxidation (6, 10). In addition, the relative antioxidant efficacies of estrogens are closely related to their molecule structure (6, 7). Catecholestrogens acted as the most powerful inhibitors of microsomal lipid peroxidation in all experimental conditions (6). The preventive effects of catecholestrogens were independent of the functional integrity of microsomes or the presence of NADPH, underlining the importance of the chemical nature in their action. Therefore, the structural modification of E_2 under its metabolization may also explain the dependence of E_2 efficacy on the activity of the cytochrome P450 system.

In conclusion, this report confirms the inhibitory effects of E_2 and 2-OHE_2 against oxidation in freshly isolated rat liver cells as experimental model. Five min exposure of E_2 was able to prevent peroxidation induced by the subsequent

in vitro addition of Fe(III)/ADP. As in other studies with cell free systems, 2-OHE$_2$ was a more effective antioxidant than E$_2$. Moreover, the modulation exerted by inhibitors of cytochrome P450 on the E$_2$ effects may be taken as evidence for the involvement of E$_2$ metabolism in its antioxidant activity.

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M. B. RUIZ-LARREA, A. M. LEAL, C. MARTÍN, R. MARTÍNEZ y M. LACORT. *Acción antioxidante de los estrógenos en hepatocitos de rata.* J. Physiol. Biochem. (Rev. esp. Fisiol.), 53 (2), 225-230, 1997.

La adición *in vitro* de 17β-estradiol (0-100 μM) a hepatocitos aislados de rata previene la peroxidación lipídica celular inducida por el complejo Fe(III)/ADP, aunque menos eficazmente que su derivado metabólico 2-hidroxiestradiol. La presencia de inhibidores específicos de la actividad del citocromo P450 disminuye la capacidad antioxidante del estradiol. Estas observaciones sugieren que el estradiol, a concentraciones micromolares, inhibe la peroxidación de lípidos inducida por hierro en las células hepáticas, debido a que desvía equivalentes de reducción del proceso peroxidativo hacia su propio metabolismo.

Palabras clave: 17β-Estradiol, Antioxidante, Peroxidación de lípidos, Hepatocitos de rata.

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